

It has been suggested that tissue plasminogen activator (tPA), which is widely used for the thrombolytic treatment of stroke, exhibits neurotoxic side effects. To test this hypothesis, mice exposed to 90 min non-thrombotic middle cerebral artery thread occlusion were treated with 10 mg/kg recombinant tPA (rt-PA) at 15 min after the onset of vascular occlusion. Measurements of blood flow, infarct volume, brain swelling and neurological performance revealed faster recirculation and a significant reduction of ischemic injury in rt-PA-treated animals. These data are at variance with previous reports on tPA neurotoxicity and demonstrate, on the contrary, that tPA protects the brain even after non-thrombotic vascular occlusion. *NeuroReport* 10:107–111 © 1999 Lippincott Williams & Wilkins.

Key words: Blood flow; Brain edema; Focal ischemia; Neurological score; Neurotoxicity; Tissue plasminogen activator (tPA); Vital staining

Recombinant tissue plasminogen activator reduces infarct size after reversible thread occlusion of middle cerebral artery in mice

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Introduction

With the increasing use of tissue plasminogen activator (tPA) for the treatment of acute stroke, concerns have been raised that the desirable benefit of thrombolysis treatment is cancelled out by undesirable side effects [1]. The most obvious complication is intracerebral bleeding, but recent evidence suggests that tPA is neurotoxic and could induce neurodegeneration after ischemia/reperfusion [2,3]. In fact, tPA is a serine protease that converts plasminogen to protease plasmin which is involved in the signalling cascade leading to excitotoxic neuronal death [3]. The relevance of this mechanism *in vivo* is supported by the fact that tPA-deficient mice are remarkably resistant to excitotoxin-induced hippocampal injury [3]. Another mechanism of tPA-induced neurodegeneration may be the up-regulation of ICAM-1 and the adhesion of neutrophils to microvascular endothelial cells [4]. Both processes may contribute to molecular events that eventually result in programmed cell death, as demonstrated in various types of brain injury [5,6].

The hypothesis of tPA-induced neurodegeneration is strongly supported by the observation that suture occlusion of the MCA in tPA-deficient mice produces infarcts about 50% smaller than those produced in wild-type mice [2]. Conversely, therapeutic application of tPA increases the volume of infarct in both tPA-deficient and wild-type animals

[2]. Obviously, a neurotoxic effect of tPA could compromise the viability of brain tissue after therapeutically induced thrombolysis and, therefore, would be of considerable clinical importance. However, experimental studies of tPA-induced thrombolysis provided no evidence of neurotoxicity but instead demonstrated a close correlation between reperfusion and tissue preservation [7]. We, therefore, reinvestigated the effect of tPA on non-thrombotic focal brain ischemia, using a retractable thread for vascular occlusion and subsequent restoration of circulation [8]. This approach makes it possible to distinguish the potentially neurotoxic effects of tPA from the beneficial effects of reperfusion which should be similar in treated and untreated animals. Our observations do not support the hypothesis of a neurotoxic effect of tPA in focal ischemia but, quite to the contrary, demonstrate a substantial reduction of ischemic injury.

Materials and Methods

All experimental procedures were carried out with governmental approval according to the NIH guidelines for the care and use of laboratory animals.

Induction of focal ischemia: Adult C57BL/6j mice weighing 21–27 g were anesthetized with 1% halothane in 30% O₂, 70% N₂O. Rectal temperature was maintained at 36.5°C using a feedback-

controlled heating system. Focal cerebral ischemia was induced by transient thread occlusion of the middle cerebral artery (MCA) for 90 min. After midline neck incision, the left common and external carotid arteries were isolated and ligated. A microvascular clip (FE691; Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery. A 8-0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicon resin (Xantopren; Bayer Dental, Osaka, Japan) was introduced through a small incision into the common carotid artery and advanced 9 mm distal to the carotid bifurcation for occlusion of the middle cerebral artery. The size of the thread (150–200 μ m) was matched to the body weight to ensure reproducible vascular occlusion [8]. After 90 min ischemia, MCA reperfusion was initiated by withdrawal of the thread. Thirty minutes later anesthesia was discontinued and animals were returned to their cages.

Laser-Doppler flowmetry: For monitoring of MCA occlusion cerebral blood flow was measured by laser-Doppler flowmetry (LDF). The tip of a flexible 0.5 mm fiberoptic probe (Perimed, Stockholm, Sweden) was fixed with tissue adhesive (Aron Alpha, Toa, Tokyo, Japan) to the intact skull overlying the territory supplied by the MCA (2 mm posterior and 6 mm lateral from bregma). Changes in cortical perfusion were monitored throughout ischemia and during the initial 30 min of reperfusion.

Treatment with rt-PA: Treated animals ($n=6$) received 10 mg/kg rt-PA (Actilyse; INN alteplase, Boehringer Ingelheim, Germany) by i.v. infusion. The drug was dissolved in 0.3 ml distilled water and infused into the femoral vein for 45 min, starting 15 min after the onset of vascular occlusion. Untreated control animals received either an i.v. infusion of 0.3 ml water (carrier-treated control group, $n=5$) or no infusion (untreated control group, $n=6$).

Neurological deficit scores: The neurological deficits were monitored 24 h after vascular occlusion using the following score [8]: 0 = normal function; 1 = flexion of torso and of the contralateral forelimb upon lifting of the animal by the tail; 2 = circling to the contralateral side but normal posture at rest; 3 = reinclination to the contralateral side at rest; 4 = absence of spontaneous motor activity.

Triphenyltetrazolium chloride (TTC) staining: Twenty-four hours after MCA occlusion animals were reanesthetized with halothane and decapitated. Brains were dissected into five equidistant slices and immediately stained with 2% 2,3,5-triphenyltetra-

lium chloride (TTC) [9]. The border between infarcted and non-infarcted tissue was outlined using an image analysis system, and the area of infarction was measured by subtracting the area of the non-lesioned ipsilateral hemisphere from that of the contralateral side. The volume of infarction was calculated by integration of the lesion areas. Edema was calculated as the volume difference between the ischemic and the non-ischemic hemisphere, and expressed as a percentage of the intact hemisphere.

Statistical evaluation: Values are given as mean \pm s.d. unless otherwise stated. Differences between treated and untreated animals were calculated by ANOVA followed by Fisher's PLSD test. The significance level was set at $p < 0.05$.

Results

All animals survived the first part of the experiment, i.e. the surgical preparation for MCA occlusion, and 90 min ischemia period and the initial post-ischemic recovery period. During the following 24 h one of the carrier-treated animals but none of the rt-PA-treated animals died. Macroscopic inspection revealed no intracerebral or subarachnoid hemorrhage in any of the untreated or rt-PA-treated animals.

The flow measurements obtained during and after 90 min transient middle cerebral artery occlusion are summarized in Fig. 1. After MCA occlusion, mean laser-Doppler flow declined to $< 20\%$ in all animal groups. In the untreated control groups, removal of the thread led to the return of flow to control levels within 10 min. In the rt-PA-treated animals, recirculation was faster, with hyperemia developing as early as 5 min after removal of the suture. After 30 min recirculation blood flow in rt-PA treated animals was 50% above normal, compared to a mean increase of only 10% in the two untreated control groups.

The acceleration of blood flow by rt-PA led to significantly smaller infarcts, as determined by vital staining of coronal brain slices 24 h after MCA occlusion (Fig. 2). In the untreated control groups, infarct volume was 104 ± 28 and 109 ± 45 mm³, respectively. The rt-PA treated group exhibited a significant ($p < 0.05$) infarct reduction by about 50% to 54 ± 32 mm³ (Fig. 3). Ischemic brain edema, expressed as percentage swelling of the ipsilateral hemisphere, was $11 \pm 1.8\%$ and $11 \pm 5.2\%$ in the control groups. Treatment with rt-PA significantly reduced swelling to $2 \pm 1.8\%$ ($p < 0.01$). The improvement of ischemic injury by rt-PA was accompanied by an improvement of neurological deficits. In the untreated control groups mean neurological deficit scores were 2.30 ± 0.44 and 2.16 ± 0.93 re-

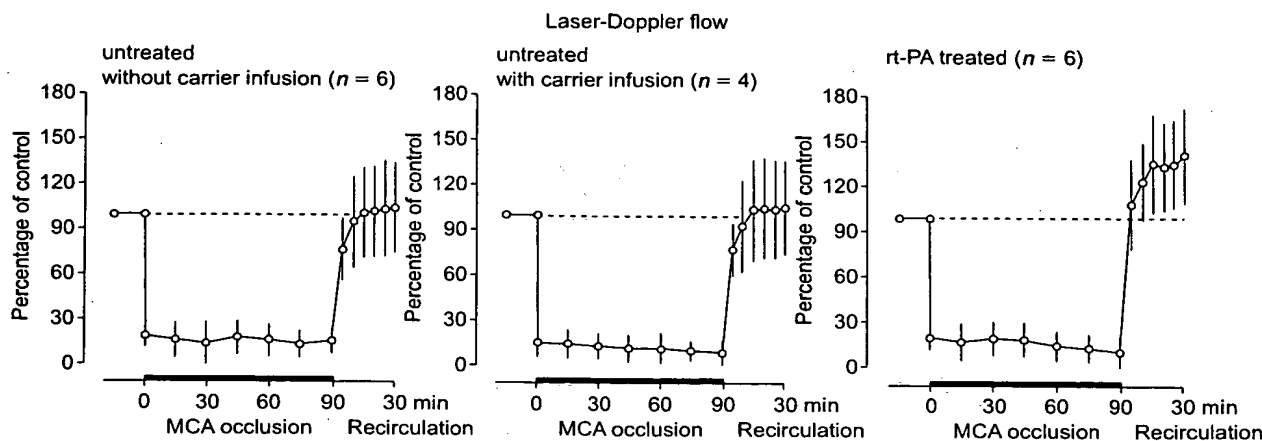


FIG. 1. Measurement of blood flow in cerebral cortex of mice during and after 90 min reversible thread occlusion of the middle cerebral artery (MCA). Comparison of untreated mice (with and without carrier infusion) with mice receiving 10 mg/kg recombinant tissue plasminogen activator (rt-PA) 15 min after the onset of vascular occlusion. Blood flow was recorded by laser-Doppler flowmetry in the territory of the occluded MCA and expressed as a percentage of the pre-ischemic value (means \pm s.d.). Note the faster restoration of blood flow and the more pronounced post-ischemic hyperemia in rt-PA treated mice than in untreated animals.

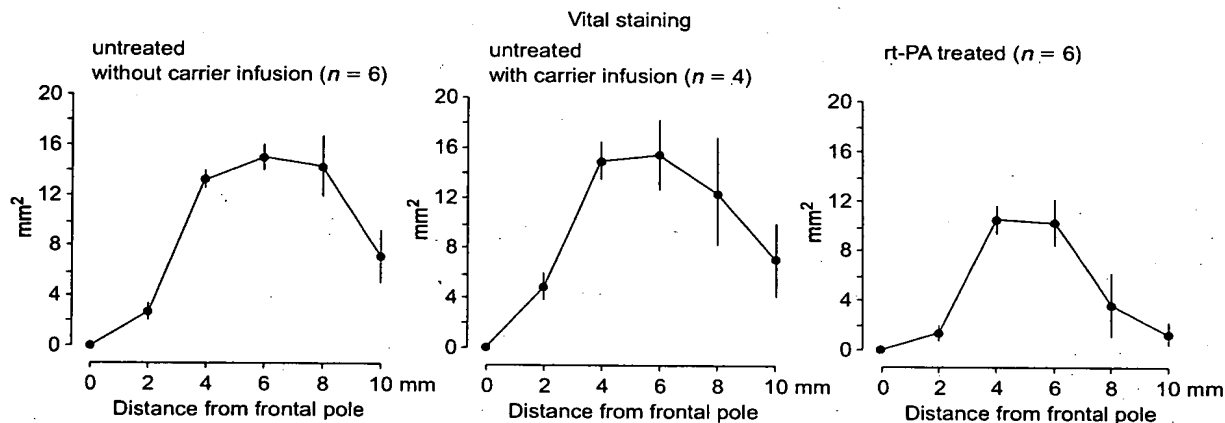


FIG. 2. Vital staining of coronal brain slices of mice 24 h after 90 min reversible MCA thread occlusion (animal groups identical to Fig. 1). Areas of infarcted tissue (means \pm s.e.m. corrected for brain swelling) were detected by absence of vital staining and plotted against the distance of slices from the frontal pole. In rt-PA treated animals infarct areas were smaller at all brain levels, indicating reduction of ischemic injury in both cortex and basal ganglia.

spectively. This score was significantly reduced to 1.16 ± 0.25 in the rt-PA-treated animals ($p < 0.05$).

Discussion

The present finding of reduced ischemic injury after rt-PA treatment is clearly at variance with the previously reported reduction of infarct volume in tPA deficient mice [2]. It is also inconsistent with the observation that rt-PA infusion increases infarct volume in both tPA-deficient and in wild-type animals [2]. These differences cannot be explained by insufficient tPA dosage, because in our series of experiments we used a 10 times higher dose than in the previous study [2] to allow for the lower thrombolytic activity of human recombinant tPA in

small rodents [10]. However, the results of the previous study may have been influenced by strain differences which in the mouse significantly affect infarct volume after MCA occlusion [11]. C57Black/6 and SV 129 mice, used for the production of mutants [2], exhibit differences in the size of the vascular territory supplied by the MCA which could partly explain the differences in infarct volume. Other methodological differences are the duration of MCA occlusion and the time of rt-PA application. In our study the duration of MCA occlusion was 90 min and treatment started 15 min after the beginning of ischemia, whereas in the previous investigation MCA occlusion lasted for 2 h and treatment began at the end of this period [2]. Although it is difficult to imagine how the more

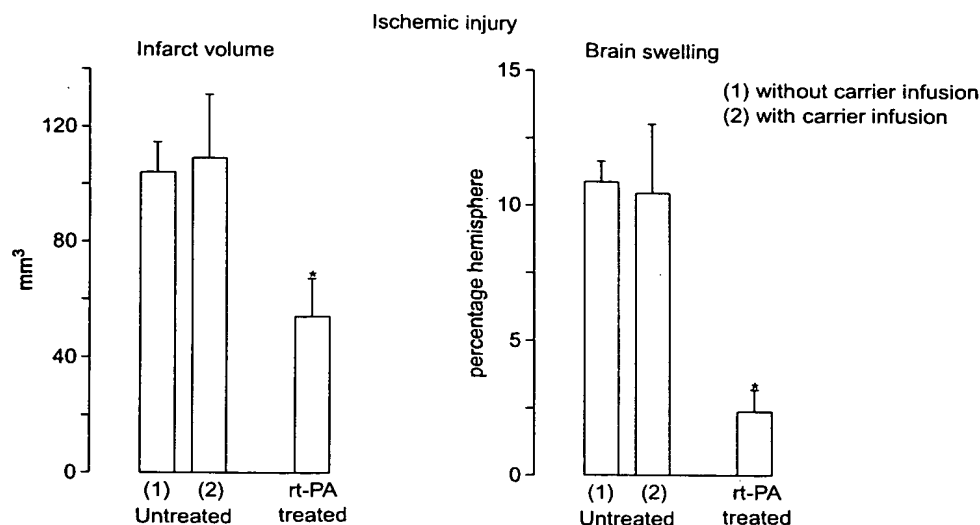


FIG. 3. Volumetric determination of brain infarcts and post-ischemic brain swelling in mice 24 h after 90 min MCA thread occlusion (animal groups identical to Fig. 1 and Fig. 2). Infarct volume (means \pm s.e.m.) was calculated by integrating swelling-corrected infarct areas shown in Fig. 2. Brain swelling (means \pm SEM) was calculated by subtracting the volume of ischemic hemisphere from that of the non-ischemic hemisphere and expressed as a percentage of the intact side. Note significant reduction of infarct volume and brain swelling after rt-PA treatment. (* $p < 0.01$).

severe primary injury, caused by the longer ischemia and the longer treatment delay, could be further aggravated by tPA-mediated neurotoxic effects, a possible relationship to the observed changes cannot be excluded.

The beneficial effect of rt-PA in our study was associated with a marked improvement of post-ischemic recirculation. The reason for the flow improvement could be either lysis of microemboli formed at the tip of the suture, or the alleviation of post-ischemic coagulopathy. Indeed, previous investigations of global ischemia revealed massive intravascular coagulation [12], probably as a consequence of complement activation [13]. It is conceivable that rt-PA settled this disturbance and thereby increased the rate of recirculation. The influence of the speed of post-ischemic recirculation on the infarct size is in line with previous observations after global ischemia which also demonstrated a close correlation between the rate of early reperfusion and the quality of post-ischemic recovery [14]. A likely explanation for this relationship is the faster restoration of the redox state of the respiratory chain and, in consequence, of energy producing metabolism under conditions of improved recirculation. Restoration of a normal tissue energy state, in turn, reactivates the ion exchange pumps, that must be functioning for the elimination of post-ischemic brain edema [15]. Factors contributing to the post-ischemic generation of reactive oxygen species, such as polymorphonuclear (PMN) leucocyte adhesion to vascular endothelial cells [16], the conversion of xanthin dehydrogenase to the oxidative form [17] or the

permeability transition of the inner mitochondrial membrane [18], are also reversed more quickly. Obviously, possibly adverse molecular side effects of tPA such as promotion of excitotoxicity [19] or the up-regulation of potentially adverse cytokines [4] are outweighed by the acceleration of metabolic recovery and are of limited pathophysiological relevance. Post-ischemic tissue injury is, therefore, less severe the faster tissue is reoxygenated, which stresses the importance of hemodynamic factors in the prevention of post-ischemic injury.

It remains to be shown, however, whether this interpretation is also valid for rt-PA-induced thrombolysis under clinical conditions. Restoration of blood flow by lysis of intravascular clot material is slower than recirculation after transient suture occlusion [7], and it is conceivable that during the critical interval between the onset of thrombolytic reperfusion and the restoration of oxidative metabolic activity, glutamate or free radical-induced molecular disturbances are of greater importance than in the experimental model described here. On the other hand, rt-PA treatment was not found to enhance edema formation as one would expect if it did promote such secondary complications. Our data, therefore, do not support the hypothesis that tPA enhances ischemia-induced brain injury.

Conclusion

Treatment with recombinant tPA after reversible thread occlusion of the middle cerebral artery in mice accelerates blood recirculation, reduces infarct

volume and brain swelling and improves neurological performance. These data are at variance with previous reports on tPA neurotoxicity [2,3] and demonstrate, on the contrary, that tPA improves brain recovery after transient vascular occlusion.

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